Utilization of MS^3 Spectra for the Multicomponent Quantification of Diastereomeric *N*-Acetylhexosamines

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A rapid and accurate means of quantifying mixtures of diastereomeric *N*-acetylhexosamine monosaccharides using MS^3 product ions is introduced. The method involves derivatizing the monosaccharides with $[Co(DAP)_2Cl_2]Cl$ (where DAP is diaminopropane), and subjecting the derivatized products to collision-induced dissociation (CID) in a quadrupole ion trap mass spectrometer. Each diastereomer provides unique MS^3 product ion abundances. The abundances for the pure monosaccharide standards are used in a system of equations in order to quantify mixtures of these diastereomers. Using the system of equations is quite advantageous, as it is the only mass spectrometric method that has been shown to successfully quantify mixtures of more than two isomers. The utility of the method is demonstrated by successfully quantifying various two and three component mixtures of the diastereomeric monosaccharides. Furthermore, the method is used to quantify the recovery of a single diastereomeric monosaccharide from an acidic resin. Although the multicomponent quantification method described herein is used to quantify mixtures of *N*-acetylhexosamine diastereomers, it could be applied to any group of isomers, provided distinguishing CID spectra are obtained. This is the first known report of utilizing MS^3 product ions for quantification of structural isomeric mixtures. (J Am Soc Mass Spectrom 2000, 11, 1086–1094) © 2000 American Society for Mass Spectrometry

Mass spectrometry is rapidly becoming the analytical technique of choice for structural characterization of biologically isolated compounds, because sample consumption requirements are minimal, and data acquisition takes only minutes [1]. However, complete structural elucidation of many biologically isolated macromolecules, including carbohydrates and proteins, is currently difficult or impossible using solely mass spectrometry due to the presence of isomeric components. This problem is particularly challenging for carbohydrate chemists, because the two most common monosaccharide units, hexoses and *N*-acetylhexosamines, each have three possible diastereomeric forms. Thus, structural elucidation of carbohydrates must include a method to determine the stereochemistry of each of these monosaccharide units [1].

Therefore, for determining the diastereomeric forms of carbohydrates, researchers typically use nonmass spectrometric methods, such as high-performance anion-exchange chromatography (HPAEC) with pulsed amperometric detection [2–4]. This approach suffers from several disadvantages; specifically, the long column regeneration time between runs and the poor reproducibility of retention times of the monosaccharides [3, 4].

Mass spectrometry has the potential for providing more rapid and more accurate analyses, while maintaining the low sample consumption requirements (20–200 pmol/μL) of HPAEC [2, 4]. Thus, several research groups have developed methods that allow for differentiation of diastereomeric monosaccharides using mass spectrometry [5–17]. In each of these cases, the stereochemistry of pure diastereomeric monosaccharides was identified based on the intensity of product ions generated [10–17] or based on the presence/absence of product ions present in CID experiments [5–9]. In applying this technique to biologically isolated carbohydrates, total acid hydrolysis must precede the identification of the stereochemistry of each of the monosaccharide components [5]. After hydrolysis, the mass spectrometry techniques can be used to identify the stereochemistry of the monosaccharides present, provided one of two conditions are met: either only one type of diastereomer may be present, or the diastereomers must be separated after the hydrolysis. These conditions are required because the mass spectrometry conditions mentioned thus far may only distinguish the stereochemistry of a single monosaccharide at a time. At least two examples exist which show that the stereochemistry of monosaccharide products (resulting from the acid hydrolysis of an oligosaccharide) can be deter-

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mined via mass spectrometry. Separation of the hydrolyzed products prior to analysis was not required because in each of these cases, only one type of diastereomer was present [5, 18]. Although these mass spectrometry techniques could also be used on an oligosaccharide that has a variety of isomeric monosaccharides present, the mass spectrometry analysis must be preceded by a chromatographic separation to purify the diastereomers a priori.

In order to overcome the limitation of analyzing a single diastereomer at a time, we have recently developed a unique procedure that can be used to analyze a mixture of diastereomers without prior separation [19]. We have recently shown that the three diastereomers $GlcNH_2$, $GalNH_2$, and $ManNH_2$ could be quantified by MS/MS using multicomponent quantification, a method that employs a system of equations to determine the exact percent of each diastereomer in solution. This quantification procedure is both facile and rapid, because only the product ion intensities of the pure monosaccharides are needed to determine the composition of two and three component mixtures of monosaccharides. Furthermore, it is unique in that it does not use calibration plots; as a result, it is the only method capable of quantifying mixtures of more than two diastereomers using electrospray ionization (ESI)-MS^n technology.

In this study, we attempted to apply the same technique to the quantification of *N*-acetylhexosamines. However, we found that the technique was not generally applicable to other classes of diastereomers. Specifically, the previous methodology [19] is limited to quantifying isomers that have the same ionization efficiency *and* are distinguishable by their MS^2 spectra. Thus, the previous technique was modified by introducing a new normalization which accomplishes several tasks: It accounts for the differences in ionization efficiency (or derivatization efficiency) for the isomers, and it allows MS^2 or MS^3 spectra to be used. Thus, the new protocol is a substantial advancement. Furthermore, with the new normalization, the technique is now generally applicable to any class of isomers with distinguishing CID spectra. We have recently demonstrated its utility for quantifying isomeric sulfated disaccharides [20] and we demonstrate its use for the quantification of three *N*-acetylhexosamines herein. Additionally, we demonstrate a new application of the method, quantifying the amount (in micrograms) of a single diastereomer recovered from an acidic resin. Although the examples of multicomponent quantification used thus far have included isomeric carbohydrates, the mass spectrometric method may be applied generally to any mixture of isomeric samples—provided unique product ion spectra of the isomers are obtainable. Equally important, this is the first report of quantification of structural isomeric mixtures using MS^3 product ions.

Experimental

Synthesis of the $[Co(DAP)_2Cl_2]Cl$ complex has been described previously [21] (DAP is diaminopropane).

Synthesis of the N-*Acetylhexosamine Complexes*

A methanolic solution of the $[Co(DAP)_2Cl_2]Cl$ complex (0.02 M) and triethylamine (0.04 M) was prepared daily. A portion of this solution (23 μL) was added to 20 μL of a 0.023 M aqueous solution of the monosaccharide. (For 2 or 3 component mixtures of monosaccharides, the total monosaccharide concentration was maintained at 0.023 M.) The reaction was heated at 57 °C for 15 min prior to dilution to 50 pmol/μL with methanol.

Recovery Experiment

The acidic resin was freshly prepared as described elsewhere [18]. The resin (0.030 g) was added to a 100 μL conical vial. The aqueous monosaccharide solutions [10 μL, 0.023 M GalNAc (50 μg) and 12 μL, 0.023 M glucose (50 μg)] were added to the resin, along with 50 μL of H_2O. The vial was capped and vortexed for 5 min. Subsequently, the resin was filtered through a 100,000 MW polysulfone microcentrifuge tube. The vial and cap (which initially contained the resin) were washed with 2×50 μL H_2O, and the washings were added to the resin. The resin (and washings) were vortexed for 2 min and refiltered through the microcentrifuge tube. The combined filtrate was then lyophilized for 2 h. To the lyophilized product, 10 μL H_2O + 10 μL of 0.023 M (aqueous) GlcNAc solution was added. After vortexing for 1 min, 23 μL of the metal solution was added, and the sample was heated as described above.

Mass Spectrometry

All the experiments described were analyzed on a quadrupole ion trap mass spectrometer fitted with an ESI source (Finnigan LCQ, purchased from Finnigan-Mat, San Jose, CA). Several parameters were deemed crucial in order to obtain reproducible CID spectra, and those parameters are as follows: The samples were diluted and analyzed immediately after derivatization, and they were injected into the mass spectrometer via direct infusion at a flow rate of 5 μL/min. (Lower flow rates produced less reproducible results.) The capillary temperature was set to 150 °C. The spray voltage was maintained at 5 kV, and the ESI probe was placed very close to the spray shield, such that the ESI probe protruded 1.6 cm from the ESI flange. Optimization of the ion of interest (*m/z* 426) was initially achieved using the automatic tuning parameter on the instrument, and this tuning file was loaded for every subsequent experiment. To avoid space charge effects, the automatic gain control was set to 5×10^7 counts for MS^1 experiments and 2×10^7 for MS^n experiments. This afforded a

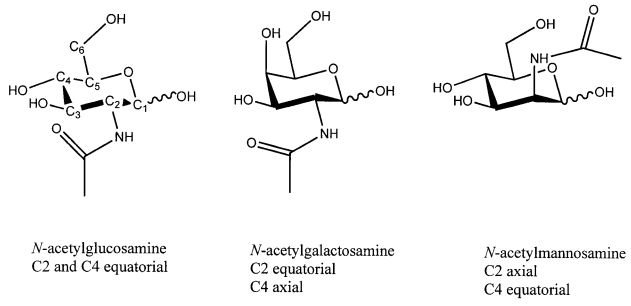

Figure 1. Diastereomeric *N*-acetylhexosamine monosaccharides.

typical ion injection time of 20 ms during MS^3 experiments and a typical ion signal of 1×10^6 counts.

During MS^n experiments, the mass range scanned was *m/z* 200 to 430. Selection of *m/z* 426 was achieved using a 5 Da window, and the ion was activated at 0.55 V (19% normalized collision energy) for 30 ms, with q_z maintained at 0.25. Selection of *m/z* 352 in the MS^3 experiment was also achieved using a 5 Da window. The ion was activated at 0.37 V (14% normalized collision energy) for 1000 ms, maintaining q_z at 0.25. Each spectrum consisted of 40 scans. The data acquisition software used in this case was Xcalibur, Version 1.0.

Quantification

Each pure monosaccharide standard was derivatized and subjected to the MS^3 experiment (described above) six times. The 1:1:1 mixture of the GlcNAc, GalNAc, and ManNAc complexes was also derivatized and analyzed six times, and every other mixture (listed in Table 5) was derivatized and analyzed once.

After all the MS^3 spectra are acquired, the "percent total ion current" of the ions of interest (*m/z* 221, 232, 262, and 322) from each spectrum is determined in the following manner: The total ion current is obtained by summing the abundance of all the ions in the mass list. The "percent of the total ion current" for each of the ions of interest is determined by finding the maximum relative abundance for each ion, and summing all values in a 1.2 Da window around the maximum relative abundance. For example, in the GlcNAc complex the product ion *m/z* 261.93 had a 100% relative abundance, so all of the values between 261.33 and 262.53 are summed. This sum is then divided by the total ion current, and the resulting quotient is the "percent total ion current," or the "contribution," of the ion. For the pure monosaccharide standards and the 1:1:1 mixtures, the contributions for each of the six analyses were averaged, as shown in Tables 2 and 4.

The "contributions" for the ions are then used in three different ways. (1) For the pure monosaccharide standards, the averaged contributions are used as constants in a system of equations (as described in the text below which accompanies the system of three equations). (2) For the 1:1:1 mixture, the averaged contributions are used to determine normalization 1 (as described in the text accompanying Table 4). (3) For every other two- or three-component mixture, the contributions are used to determine the composition of the mixture (as described in the text accompanying Table 5).

Results and Discussion

The three diastereomeric *N*-acetylhexosamines, *N*-acetylglucosamine (GlcNAc), *N*-acetylgalactosamine (GalNAc), and *N*-acetylmannosamine (ManNAc) are depicted in Figure 1. Previously, we showed that unique product ion spectra are generated when these three diastereomeric *N*-acetylhexosamines are derivatized with $[Co(DAP)_2Cl_2]Cl$ and allowed to undergo CID [5]. Thus, the identity of each diastereomer could be uniquely determined based on the presence or absence of product ions observed in the MS^2 and MS^3 spectra (Table 1). Likewise, the origin of each these product ions has been determined by isotopic labeling experiments performed earlier [5], and the composition of each loss is indicated in Table 1. Although these spectra are quite useful in identifying a single, unknown diastereomer, they are not sufficient to quantify mixtures of the diastereomers, because at least one distinguishing ion must be present for each component. In the MS^2 data, there is no distinguishing product ion for the GalNAc complex, and similarly, the MS^3 data for the ManNAc complex shows no distinguishing ions present (Table 1). Furthermore, both of the distinguishing ions in the MS^2 spectra are rather small, approximately 13% and 20% relative abundance for *m/z* 295 and

Table 1. Comparison of dissociations for the $[Co(Dap)_2(HexNAc - 2H)]^+$ complex

	Product ions detected[a]					
	MS^2 results			MS^3 results		
	m/z 295 $-C_5H_9NO_3$	*m/z* 306 $-C_4H_8O_4$	*m/z* 310 –DAP	*m/z* 262 $-C_3H_6O_3$	*m/z* 292 $-C_2H_4O_2$	*m/z* 322 $-CH_2O$
GlcNAc	++	++	++	++	++	++
ManNAc	−−	++	++	−−	−−	−−
GalNAc	−−	−−	++	−−	++	++

[a] "++" means the ion is present in the CID spectra. "−−" means the ion is absent.

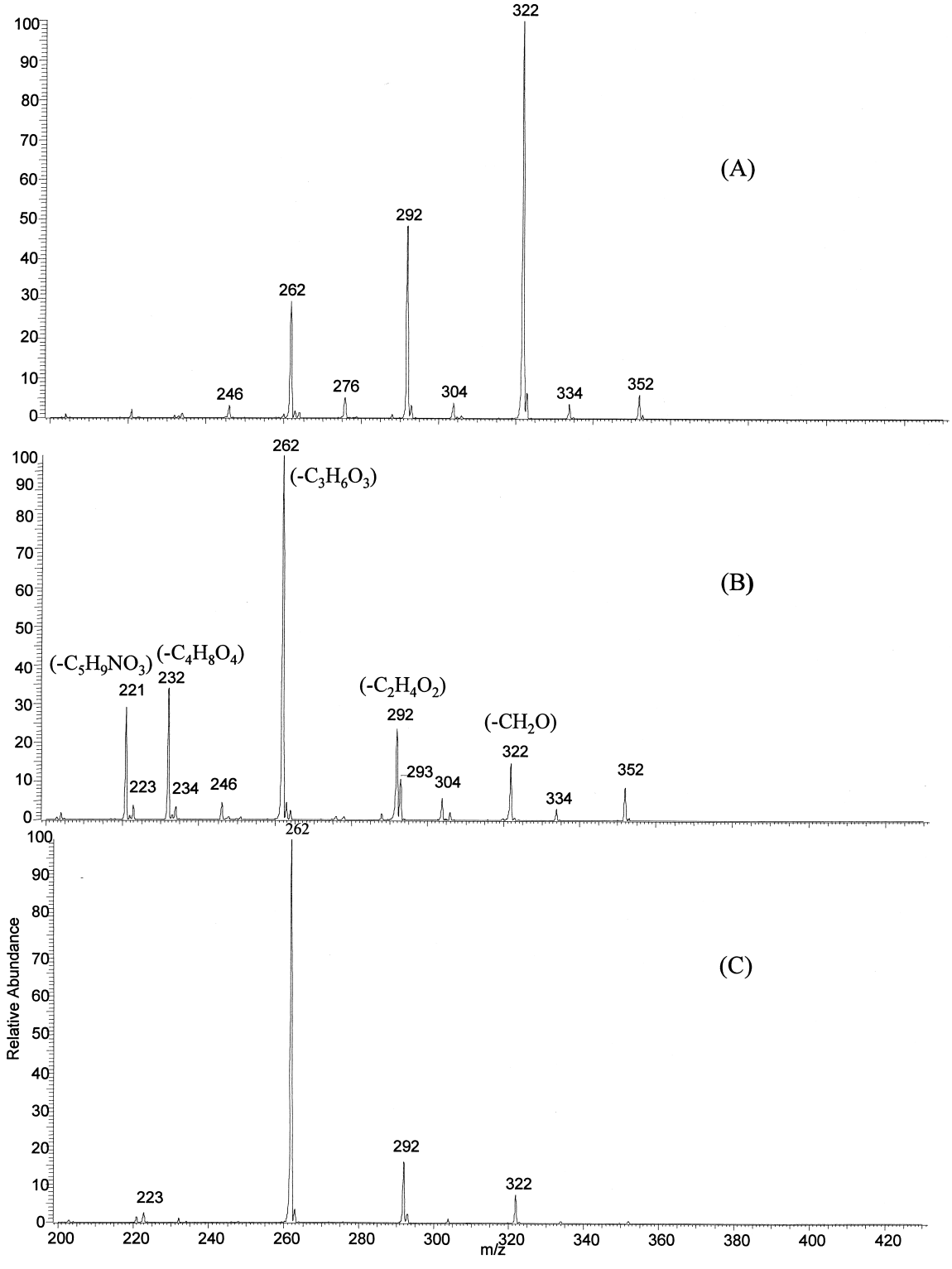

Figure 2. MS^3 spectra (*m/z* 426 → 352 →) of the three diastereomeric complexes: (**A**) $[Co^{III}(DAP)(GalNAc - 2H)]^+$, (**B**) $[Co^{III}(DAP)(ManNAc - 2H)]^+$, and (**C**) $[Co^{III}(DAP)(GlcNAc - 2H)]^+$. The ion at *m/z* 426 represents the octahedral complex $[Co^{III}(DAP)_2(HexNAc - 2H)]^+$, and the ion *m/z* 352 (used in the MS^3 experiment) represents a loss of one DAP ligand from this complex.

306, respectively. Thus, in order to quantify the three diastereomers, sufficiently discriminating product ion spectra must be obtained.

Quantitatively useful spectra are obtained when the collision energy is increased during the MS^3 experiment. Previously, the MS^3 experiment (*m/z* 426 → *m/z*

Table 2. Reproducibility of contributions for monosaccharide standards

		Contributions for each ion (% of TIC)							
		1	2	3	4	5	6	Average	STDEV
GlcNAc	% *m/z* 221	1.00	1.01	0.86	0.82	0.85	1.10	0.94	0.11
	% *m/z* 232	0.55	0.53	0.48	0.54	0.61	0.43	0.52	0.06
	% *m/z* 262	69.65	69.38	70.13	70.59	69.59	70.49	69.97	0.51
	% *m/z* 322	5.17	5.61	4.42	4.26	4.75	4.26	4.75	0.55
ManNAc	% *m/z* 221	10.16	10.01	10.44	10.43	10.15	9.99	10.20	0.20
	% *m/z* 232	12.10	12.16	12.50	12.80	12.33	12.39	12.38	0.25
	% *m/z* 262	35.96	35.19	35.58	35.39	34.83	34.87	35.31	0.43
	% *m/z* 322	5.86	6.58	5.74	5.73	7.07	6.52	6.25	0.55
GalNAc	% *m/z* 221	1.01	0.76	0.79	1.04	1.05	1.13	0.96	0.15
	% *m/z* 232	0.24	0.24	0.25	0.26	0.27	0.21	0.24	0.02
	% *m/z* 262	12.40	12.04	12.52	12.83	13.16	13.49	12.74	0.53
	% *m/z* 322	44.59	45.76	44.11	43.73	44.12	43.53	44.31	0.80

352 →) was run at lower collision energy (0.25 V) [5] such that the diastereomers could be distinguished based on their presence or absence of product ions. By increasing the collision energy to 0.37 V, the spectra are more suitable for quantitative purposes (Figure 2). At higher collision energy, each diastereomeric complex produces "distinguishing" product ions, which have substantially different abundances compared to other isomeric species. For example, in the MS^3 spectrum in Figure 2A, the GalNAc complex has a distinguishing product ion at *m/z* 322, which is in much greater abundance than that of the GlcNAc and ManNAc complexes (Figure 2B, C). Furthermore, in the higher energy regime, the ManNAc complex now has two distinguishing product ions associated with it, *m/z* 232 and 221. The distinguishing product ion for the GlcNAc complex is *m/z* 262. Although *m/z* 262 is also the base peak for ManNAc, the percent total ion current for *m/z* 262 is only 35% for ManNAc, compared to 70% for GlcNAc (Table 2). Although the monosaccharides are no longer distinguishable based on presence or absence of product ions as they were before, unique product ions are not required for quantification, because the relative abundance of each product ion is quite reproducible.

Conditions which afford highly reproducible spectra have been described previously [19] and are briefly reiterated in the experimental section. Table 2 shows the reproducibility of the product ions obtained for each monosaccharide complex. In each case, the contribution of the four distinguishing ions (*m/z* 221, 232, 262, and 322) is reported as a percent of the total ion current (see Experimental). Excellent reproducibility is observed, with standard deviations of less than 1%. This level of reproducibility has never been demonstrated for MS^3 spectra.

According to the method described previously [19], after obtaining the contribution of each distinguishing product ion (for the pure monosaccharide standards), these contributions are used in the following system of equations:

$$a \times 221_{glc} + b \times 221_{man} + c \times 221_{gal} = C_{221}$$

$$a \times 262_{glc} + b \times 262_{man} + c \times 262_{gal} = C_{262}$$

$$a \times 322_{glc} + b \times 322_{man} + c \times 322_{gal} = C_{322}$$

In this system of equations, the variables a, b, and c represent the quantities of GlcNAc, ManNAc, and GalNAc respectively in any given mixture. The constants 221_{glc}, 221_{man}, and 221_{gal} are the contributions for the *m/z* 221 product ion for the pure GlcNAc, ManNAc, and GalNAc standards, respectively. Similarly, the constants 262_{glc}, 262_{man}, and 262_{gal} are the contributions for the *m/z* 262 product ions for the pure standards, and the constants 322_{glc}, 322_{man}, and 322_{gal} are the contributions for the *m/z* 322 product ions for the pure standards. Finally, the variables (C_{221}, C_{262}, and C_{322}) are the contributions measured (for the ions *m/z* 221, 262, and 322) in a spectrum where the amounts of GlcNAc, ManNAc, and GalNAc (a, b, and c) are unknown. [Note, in this system of equations *m/z* 221 is used as a distinguishing product ion for ManNAc. One could have used *m/z* 232 or the sum (*m/z* 221 + *m/z* 232) instead. The selection of *m/z* 221 will be discussed later.]

After the system of equations is generated, mixtures of the monosaccharides are analyzed. For example, a mixture of 33.3% GlcNAc, 33.3% ManNAc, and 33.3% GalNAc was derivatized with $[Co(DAP)_2Cl_2]Cl$, and injected into the ion trap. The MS^3 experiment 426 → 352 → was conducted as described above and the contribution of the three product ions, *m/z* 221, 262, and 322 were measured as a percent of total ion current. These contributions were then inserted into the system of equations as the variables C_{221}, C_{262}, and C_{322}. Solving the equations for a, b, and c (using Mathematica Version 3 for Unix) gives the unnormalized percent of each monosaccharide in the mixture (Table 3, "Raw %'s from Mathematica"). Normalizing the data so the %GlcNAc + %GalNAc + %ManNAc = 100% provides the calculated percents (Table 3, "Calculated %'s after normalization"). Upon completion of this step, the

Table 3. Calculated %'s for the 1:1:1 mixture (using only 1 normalization)

	Contribution for each ion (% TIC)			Raw %'s from Mathematica			Calc. %'s after normalization		
	m/z 221	*m/z* 262	*m/z* 322	GlcNAc	ManNAc	GalNAc	GlcNAc	ManNAc	GalNAc
Sample 1	3.40	35.01	22.69	28.6	26.5	44.4	28.7	26.7	44.6
Sample 2	3.31	34.39	23.77	27.7	25.5	47.1	27.7	25.4	47.0
Sample 3	3.29	34.97	23.18	28.9	25.3	45.7	28.9	25.3	45.7
Sample 4	3.44	34.71	22.97	27.8	26.9	45.1	27.9	27.0	45.2

calculated percents should be 33:33:33; however, the calculated values are clearly inaccurate. The 33:33:33 mixture was remade and reanalyzed several times (Table 3, lines 1–4). Each time, the calculated percents were quite precise, but inaccurate.

It is clear that the multicomponent quantification procedure as described earlier [19] is not accounting for some variable that is present in this assay. This new variable may result from several possible sources. For example, because the diastereomers are derivatized prior to analysis, it is likely that this derivatization is not 100% efficient for all the diastereomers. It is quite likely that the derivatization does not occur to the same extent for each of the diastereomers, either. There may be a larger steric barrier to metal–carbohydrate binding for ManNAc, because ManNAc is the only monosaccharide with an axial *N*-acetyl group. Thus, if less of the $Co(DAP)_2(ManNAc - 2H)^+$ complex is formed initially, the "calculated percent" of ManNAc will be systematically too low; or similarly, the ionization efficiencies of the complexes may not be identical.

Other factors that influence the results in Table 3 may also be involved. For example, in these experiments MS^3 spectra are being used, not MS^2 spectra (which were used in the previous study [19]). It is likely that all three diastereomeric complexes are not retained equally in the ion trap during the isolation of *m/z* 352. During CID of *m/z* 426, GlcNAc and ManNAc both produce product ions other than *m/z* 352 (Table 1); whereas CID of *m/z* 426 for the GalNAc complex only produces the product ion *m/z* 352. Thus, it is reasonable to expect that the population of *m/z* 352 is biased towards GalNAc, even if the composition of all three diastereomeric complexes starts out equal.

A new normalization factor is introduced which accounts for the all of the effects described above. The normalization factor is obtained in the following manner: The 1:1:1 mixture is synthesized and analyzed a total of six times (as were the three pure monosaccharide standards), and the average contribution for the ions *m/z* 221, 232, 262, and 322 are tabulated (Table 4). The average contributions for *m/z* 221 (3.37%), 262 (34.92%), and 322 (23.13%) are input into the Mathematica program, and values for *a*, *b*, and *c* (raw %'s of GlcNAc, ManNAc, and GalNAc) are determined. These raw %'s are 28.4%, 25.5%, and 46.2% for GlcNAc, ManNAc, and GalNAc, respectively. The percents are subsequently divided by constants such that %GlcNAc = %ManNAc = %GalNAc = 33.3%. Those constants are 0.851, 0.765, and 1.39 for GlcNAc, ManNAc, and GalNAc, respectively. Henceforth, these constants will be referred to as normalization 1. (Note that regardless of the composition of the mixture analyzed, the constants for normalization 1 never deviate from 0.851, 0.765, and 1.39.)

To demonstrate the effectiveness of multicomponent quantification using the new normalization procedure, a variety of two and three component mixtures were analyzed, and the percent of each monosaccharide in the mixtures, calculated (Table 5). The analysis of sample 1, Table 5, will be used to demonstrate each step of the new quantification procedure. The sample is derivatized with the metal–ligand system, and allowed to undergo CID. The contributions of the ions *m/z* 221, 262, and 322 are measured. For sample 1, they are 1.0%, 20.1%, and 39.4% for *m/z* 221, 262, and 322, respectively (Table 5, sample 1, "% of TIC for ions"). These values are then entered into the Mathematica program, and the raw %'s of GlcNAc, ManNAc, and GalNAc are recorded (12.6, 0.4, and 87.6, respectively) (Table 5, sample 1, "Raw %'s from Mathematica"). The raw percents are normalized by dividing %GlcNAc by 0.851, dividing %ManNAc by 0.765, and dividing %GalNAc by 1.39, giving values of 14.8, 0, and 63.2 for GlcNAc, ManNAc, and GalNAc, respectively (Table 5, sample 1, "%'s after norm. 1"). Any monosaccharide with less than 2% contribution is determined to be absent (set to 0%). The "%'s after normalization 1" are subsequently

Table 4. Reproducibility of contributions for the 1:1:1 mixture

	Contributions for each ion (% TIC)							
	1	2	3	4	5	6	Average	STDEV
% *m/z* 221	3.40	3.31	3.29	3.44	3.40	3.02	3.37	0.07
% *m/z* 232	3.53	2.97	3.57	3.60	3.64	3.39	3.46	0.28
% *m/z* 262	35.01	34.39	34.97	34.71	35.52	33.85	34.92	0.42
% *m/z* 322	22.69	23.77	23.18	22.97	23.06	24.76	23.13	0.40

Table 5. Quantification of two and three component mixtures

	% of TIC for ions			Raw %'s From Mathematica			%'s after norm. 1			Norm. 2	Calc. %'s (after norm. 2)			Actual %'s			Difference (calc. − actual)		
Sample	*m/z* 221	*m/z* 262	*m/z* 322	GlcNAc	ManNAc	GalNAc	GlcNAc	ManNAc	GalNAc		GlcNAc	ManNAc	GalNAc	GlcNAc	ManNAc	GalNAc	GlcNAc	ManNAc	GalNAc
1A	1.00	20.14	39.42	12.6	0.4	87.6	14.8	0.0	63.2	78.0	19.0	0.0	81.0	20		80	−1.0		1.0
1B	1.04	19.95	39.03	12.3	0.9	86.6	14.4	0.0	62.5	77.0	18.7	0.0	81.3	20		80	−1.3		1.3
2	8.98	45.16	5.65	28.6	71.3	−0.4	33.1	100.4	0.0	133.4	24.8	75.2	0.0	25	75		−0.2	0.2	
3	0.31	29.03	33.48	28.3	−0.1	72.5	32.7	0.0	50.0	82.8	39.6	0.0	60.4	40		60	−0.4		0.4
4	5.68	54.65	5.03	56.3	43.5	−0.8	65.1	61.2	0.0	126.3	51.5	48.5	0.0	50	50		1.5	−1.5	
5	0.43	59.92	11.85	82.5	−0.3	17.9	95.4	0.0	12.4	107.8	88.5	0.0	11.5	90		10	−1.5		1.5
6	6.29	24.67	24.64	1.2	49.8	48.4	0.0	70.1	33.4	103.5	0.0	67.7	32.3		66.67	33.33		1.0	−1.0
7	2.61	16.19	37.62	−0.9	17.9	82.5	0.0	23.4	59.5	83.0	0.0	28.2	71.8		25	75		3.2	−3.2
8	5.95	34.14	19.44	18.9	46.6	35.3	21.8	65.5	24.3	111.7	19.5	58.7	21.8	20	60	20	−0.5	−1.3	1.8
9	3.48	51.57	11.92	57.8	25.4	17.1	66.8	35.7	11.8	114.3	58.4	31.2	10.3	60	30	10	−1.6	1.2	0.3
10	0.94	18.59	38.93	8.2	5.6	86.2	9.4	7.9	59.5	76.7	12.3	10.2	77.5	10	10	80	2.3	0.2	−2.5
11	2.30	41.10	20.72	43.4	16.0	39.9	50.2	22.5	27.5	100.2	50.1	22.4	27.4	50	20	30	0.1	2.4	−2.6
12	4.00	29.12	26.10	16.6	30.6	52.8	19.1	43.0	36.4	98.6	19.4	43.6	36.9	20	40	40	−0.6	3.6	−3.1
std	3.33	34.90	23.42	28.4	25.5	46.2	33.3	33.3	33.3	100.0	33.3	33.3	33.3	33.33	33.33	33.33	0.0	0.0	0.0

divided by normalization 2, which accounts for the fact that the %GalNAc + %GlcNAc + %ManNAc must equal 100%. For sample 1, normalization 2 is the sum of 14.8 + 0 + 63.2, or 78.0. By dividing each monosaccharide by normalization 2, the calculated percents are obtained (Table 5, sample 1, %'s after norm. 2). The calculated percents for sample 1 (19.0% and 81.0%) agree quite well with the actual percents, 20% GlcNAc to 80% GalNAc.

The modified quantification procedure works quite effectively for any two or three component mixture (Table 5). The error for all of the samples was very small (<4%), and the quantification was effectively achieved over a broad range of sample compositions. In Table 5, the ions at *m/z* [221, 262, and 322] were used. Using these ions produced better results than using other possible sets of ions. That is, one could have used the set of ions at *m/z* 232, 262, and 322 or the set of ions at *m/z* (221 + 232), 262, and 322 (Table 6). However, these sets of ions gave less accurate results than when using the ions at [*m/z* 221, 262, and 322] (Table 5). By comparing the standard deviations for *m/z* 221 and 232 in the pure monosaccharides (Table 2), it is not readily apparent why using *m/z* 221 gave more accurate results than using *m/z* 232. However, it is important to note that choosing the *best* ion, *m/z* 221, gave better results than using *both* distinguishing ions (*m/z* 221 + *m/z* 232). When more than one distinguishing ion is present for a given diastereomer, researchers may need to determine experimentally which ion provides the most accurate results, particularly if the ions are of approximately the same abundance, and the pure standards show similar standard deviations for those ions.

We have demonstrated that multicomponent quantification can be used to analyze mixtures of diastereomers. However, this technique can also be used to quantify the exact amount (in grams) of a single monosaccharide present. For example, in a newly developed partial acid hydrolysis procedure, an oligosaccharide is hydrolyzed under mild conditions using a polymeric acid resin [18]. The carbohydrate hydrolysate is recovered from the resin by centrifuging the mixture through a 100,000 MW microcentrifuge tube. Although very encouraging results have been achieved using this hydrolysis technique, the percent recovery of the monosaccharide (through the microcentrifuge tube) is unknown. This percent recovery of a single monosaccharide is easily determined using multicomponent quantification.

A "mock" hydrolysate (50 μg glucose, 50 μg GalNAc) was added to the acid resin and then removed from the acid resin by filtering the sample through the microcentrifuge tube. The amount of GalNAc recovered was quantified in the following fashion: after filtration, the sample was lyophilized, and 1.0 equivalent of GlcNAc was added. The resulting saccharide mixture was derivatized with the metal complex. MS^3 spectra were generated, and the contribution of the ions *m/z* 221, 262, and 322 were measured (Table 7). As described

Table 6. Error calculated using *m/z* 232 or (*m/z* 232 + 221)

Actual %'s			Data obtained using *m/z* 232, 262, and 292						Data obtained using *m/z* (232 + 221), 262, and 292					
			Calculated %'s			Error			Calculated %'s			Error		
Glc-NAc	Man-NAc	Gal-NAc	Glc-NAc	Man-NAc	Gal-NAc	Glc-NAc	Man-NAc	Gal-NAc	Glc-NAc	Man-NAc	Gal-NAc	Glc-NAc	Man-NAc	Gal-NAc
20	0	80	18.6	0.0	81.4	−1.4		1.4	18.7	0.0	81.3	−1.3		1.3
25	75	0	26.8	73.2	0.0	1.8	−1.8		26.7	73.3	0.0	1.7	−1.7	
40	0	60	38.6	0.0	61.4	−1.4		1.4	38.9	0.0	61.1	−1.1		1.1
50	50	0	53.0	47.0	0.0	3.0	−3.0		53.4	46.6	0.0	3.4	−3.4	
90	0	10	88.2	0.0	11.8	−1.8		1.8	88.2	0.0	11.8	−1.8		1.8
0	67	33	0.0	65.5	34.5		−1.2	1.2	0.0	65.2	34.8		−1.5	1.5
0	25	75	0.0	27.3	72.7		2.3	−2.3	0.0	27.7	72.3		2.7	−2.7
20	60	20	19.8	56.8	23.4	−0.2	−3.2	3.4	20.1	56.3	23.5	0.1	−3.7	3.5
60	30	10	60.6	28.4	11.0	0.6	−1.6	1.0	60.2	28.8	11.0	0.2	−1.2	1.0
10	10	80	13.3	6.6	80.1	3.3	−3.4	0.1	12.5	8.1	79.4	2.5	−1.9	−0.6
50	20	30	52.4	18.5	29.1	2.4	−1.5	−0.9	51.4	19.7	28.9	1.4	−0.3	−1.1
20	40	40	20.7	39.7	39.6	0.7	−0.3	−0.4	20.0	41.1	38.9	0.0	1.1	−1.1
33	33	33	33.3	33.3	33.3	0.0	0.0	0.0	33.3	33.3	33.3	0.0	0.0	0.0

earlier, the contributions of these ions were then used to determine the calculated percents of each of the diastereomers detected (Table 7, "calculated %'s after norm. 2"). By simply dividing the %GalNAc (48.8) by %Glc NAc (51.2), the %GalNAc recovered (95.3) can be calculated (Table 7, sample A, "%GalNAc recovered"). Knowing the percent recovered allows one to calculate the number of micrograms: 47.7 and 48.6 μg for samples A and B, respectively.

A simple control experiment was run to demonstrate that the presence of glucose in the mock hydrolysate sample did not effect the quantification. For the control experiment, sample 1B, Table 5, was prepared identical to sample 1A, Table 5, except 50 μg of glucose was added prior to derivatization. As evidenced by the close agreement between the "calculated %'s" for samples 1A and 1B, the addition of glucose had no effect on the accuracy of the quantification. This experiment not only corroborates the accuracy of the recovery experiment, it also demonstrates that other impurities—even those which can bind to the metal–ligand system—do not need to be separated prior to quantification.

Conclusions

Mixtures of *N*-acetylhexosamines were quantified using an improved version of the multicomponent quantification method described previously [19]. In this example, MS^3 spectra were used for the first time to quantify a mixture of isomers. Furthermore, the original quantification procedure was improved by the introduction of a new normalization factor that accounts for differences in ionization efficiency, derivatization efficiency, and overall detection efficiency of the three diastereomers. With the addition of this normalization factor, the method appears to be generally applicable to any class of isomers, as shown by our recent application of the method to a class of (nonderivatized) sulfated disaccharides [20]. Finally, we determined that choosing *the best* ion to represent each of the diastereomers is more effective than using *every* distinguishing ion.

The derivatization and analysis method presented here is an effective way of quantifying diastereomeric monosaccharides because the derivatization is facile and inexpensive; the analysis is rapid, accurate, and orthogonal separation techniques are not required. Furthermore, the method maintains its accuracy even when other types of monosaccharides are present. Not only can diastereomeric mixtures be analyzed, but the method may also be used to quantify the recovery of a single diastereomer. The multicomponent quantification protocol is the only quantification technique using ESI-MS^n which does not require generation of calibration plots, and it is the only mass spectrometry technique which quantifies mixtures of more than two isomers [19]. Most importantly, whereas the method was applied to the quantification of three diastereomeric monosaccharides, multicomponent quantification

Table 7. Quantifying the recovery of GalNAc

	% of TIC			Raw %'s (Mathematica)			%'s after norm. 1		Calculated %'s (after norm. 2)		% GalNAc recovered	μg GalNAc recovered
	m/z 221	*m/z* 262	*m/z* 322	Glc-NAc	Man-NAc	Gal-NAc	Glc-NAc	Gal-NAc	Glc-NAc	Gal-NAc		
Sample A	0.88	35.85	29.56	40.3	−1.0	62.5	47.3	45.1	51.2	48.8	95.3	47.7
Sample B	0.95	35.10	29.13	39.0	−0.1	61.6	45.7	44.4	50.7	49.3	97.1	48.6

could be performed on any group of isomers, provided sufficiently different product ion spectra are obtained.

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References

1. Geyer, H.; Geyer, R. *Acta Anat.* **1998,** *161,* 18.
2. Cataldi, T. R. I.; Campa, C.; Angelotti, M.; Bufo, S. A. *J. Chromatogr. A* **1999,** *855,* 539.
3. Landberg, E.; Lundblad, A.; Pahlsson, P. *J. Chromatogr. A* **1998,** *814,* 97.
4. Yokota, H.; Mori, K.; Yamaguchi, H.; Kaniwa, H.; Saisho, N. *J. Pharm. Biomed. Anal.* **1999,** *21,* 767.
5. Desaire, H.; Leary, J. A. *Anal. Chem.* **1999,** *71,* 1997.
6. Gaucher, S. P.; Leary, J. A. *Anal. Chem.* **1998,** *70,* 3009.
7. Smith, G.; Leary, J. A. *J. Am. Chem. Soc.* **1998,** *120,* 13046.
8. Smith, G.; Pedersen, S. F.; Leary, J. A. *J. Org. Chem.* **1997,** *62,* 2152.
9. Smith, G.; Leary, J. A. *J. Am. Chem. Soc.* **1996,** *118,* 3293.
10. New, A. P.; Haskins, N. J.; Games, D. E. *Rapid Commun. Mass Spectrom.* **1993,** *7,* 1099.
11. Tu, Y. P.; Yang, G. D.; Chen, Y. Z. *Org. Mass Spectrom.* **1991,** *26,* 645.
12. Tu, Y. P.; Chen, Y. Z.; Wang, M. L.; Jing, Z. Z. *Org. Mass Spectrom.* **1990,** *25,* 9.
13. Meyerhoffer, W. J.; Bursey, M. M. *Biomed. Environ. Mass Spectrom.* **1989,** *18,* 801.
14. Guevremont, R.; Wright, J. L. C. *Rapid Commun. Mass Spectrom.* **1988,** *2,* 47.
15. Guevremont, R.; Wright, J. L. C. *Rapid Commun. Mass Spectrom.* **1988,** *2,* 50.
16. Wang, G.; Sha, Y.; Xu, X.; Pan, J. *Anal. Chem.* **1985,** *57,* 2283.
17. Rose, M. E.; Longstaff, C.; Dean, P. G. *Biomed. Environ. Mass Spectrom.* **1983,** *10,* 512.
18. Cancilla, M. T.; Gaucher, S. P.; Desaire, H.; Leary, J. A. *Anal. Chem.* **2000,** *72,* 2901.
19. Desaire, H.; Leary, J. A. *Anal. Chem.* **1999,** *71,* 4142.
20. Desaire, H.; Leary, J. A. *J. Am. Soc. Mass Spectrom.*, **2000,** *11*(10), 916.
21. Bailar, J. C.; Work, J. B. *J. Am. Chem. Soc.* **1946,** *68,* 232.